Influence of Calcium Hydroxide Solution in RF Plasma on Sterilization of Bacterial Spores

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A low-pressure RF plasma powered by a 13.56 MHz RF power supply was generated with oxygen or calcium hydroxide solution vapor. The plasma was divided into three phases during one sterilization cycle. In the 1st phase, CCP plasma is generated in the chamber and the pressure of pure oxygen varies from 3 Pa to 100 Pa. Electron density and electron temperature are approximately 10^9 cm^{-3} and 2 eV respectively. Calcium hydroxide solution vapor injection varies the pressure from 3 Pa to 100 Pa twice during the 2nd phase of sterilization cycle. The 3rd phase operating with oxygen gas supply is as same as the 1st phase. This kind of three phase characteristic plasma with additive calcium hydroxide solution had been applied to investigate the different resistance of two commercial biological indicators i.e. the spores of *B. atrophaeus* and *G. stearothermophilus*. The survival curve obtained from experiment indicates that the former shows stronger resistance than the latter in the initial oxygen plasma sterilization phase. On the other hand, the latter shows stronger resistance to calcium hydroxide solution vapor plasma. A comparison analysis of the survival curves and optical emission spectral data from the plasma demonstrates that hydroxyl radicals play a significant role in inactivation of *B. atrophaeus* spores and eventually lead successful sterilization after the 80 minute cycle. The results observed under a scanning electron microscope show apparent desorption of *B. atrophaeus* spores after calcium hydroxide solution vapor plasma treatment.

Keywords: RF plasma, calcium hydroxide, solution vapor, sterilization cycle, survival curve.

1. Introduction

As an emerging sterilization method, low-temperature sterilization using plasma is attracting many interests nowadays. These methods utilize various kinds of gaseous as discharging medium to generate UV photons, radicals and other reactive species. These high-energy species can complete disinfections or sterilization of heat-sensitive subjects at relatively low temperature (under 60℃). On the other hand, due to the diversification of plasma condition and the limitation of surface interaction, the validations for plasma sterilization remain very difficult. The ISO standards for conventional sterilization such as steam autoclave, ethylene oxide (EtO) gas, dry heat etc. are available for plasma sterilization at present. Compared with the former, it is much more difficult to perform parametric release of sterilizers. Thus, so far, most of the sterilizers' validation or revalidation relies on biological indicator. Many publications concerning plasma sterilization using *Bacillus* spores as biological indicator had revealed that *B. atrophaeus* (ATCC#9372) (formerly defined as *B. subtilis*) and *G. stearothermophilus* show different resistance to various kinds of plasma. On the other hand, ISO standard for validation of sterilizer requires the application of biological indicator, which shows the strongest resistance to the sterilization method. Therefore, it is necessary to investigate the property of these two kinds of biological indicators under low-temperature plasma treatment.

Gram-positive bacteria like *Bacillus* cells can form metabolically dormant spore, which possess a remarkable resistance to heat, radiation, toxic chemicals etc. This kind of environmental resistance of spore is mainly attributed to a multi-layer spore structure [1,2]. The architecture and chemical features of spore coat protein have been studied extensively. It had been reported that the coat protein contains both structure resembling peptidoglycan and alkali-soluble protein [1-3]. Until now, many investigators had reported the distinct endurance of *B. atrophaeus*

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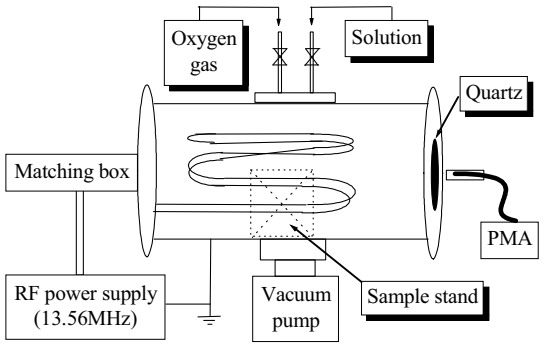

Fig.1 Schematic diagram of the experimental setup.

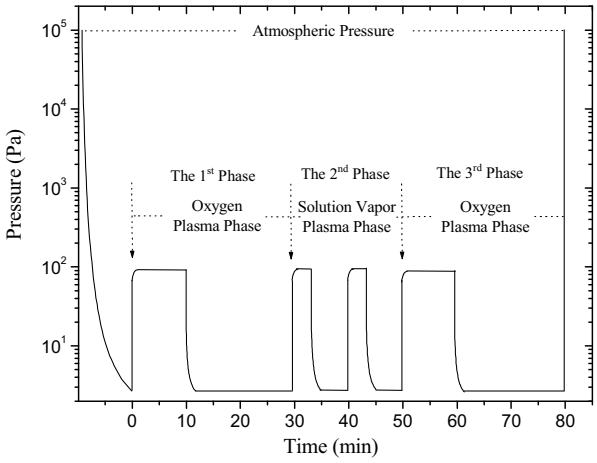

Fig.2 Sterilization cycle with three plasma phases.

spores on various sterilization methods including plasma and plasma-based sterilization [4-6]. We consider the different amino acid composition of spore coat proteins as one reason. In *B. atrophaeus* spores, there are relatively lesser peptide chain consisted of glutamic acid. On the other hand, the compositions of amino acid forming the bonds between cross-links in peptidoglycan of spore coat are considerable in *B. atrophaeus* spores. These cross-links bonds are relatively unreactive, nevertheless, the carbonyl double bond can be broken by electronegative atom. Aiming to achieve a humid environment and electronegative radical like hydroxyl, we applied calcium hydroxide solution to generate water solution vapor plasma. In this work, the influence of calcium hydroxide solution on RF plasma was investigated through spectroscopic analysis of the plasma light emission. Moreover, the sterilization efficiency of solution vapor plasma was estimated with biological indicator.

2. Experimental Procedure

The schematic diagram of experiment is shown in Figure 1. A cylinder stainless chamber, which has 200 mm in diameter and 450 mm in length, has been utilized as the plasma sterilization chamber. In order to generate plasma with higher spatial uniformity of electron density (n_e), the RF antenna wrapped with insulation coating is shaped hemi-symmetrically in the chamber. When the RF power is fixed at 40 W and the pressure of oxygen maintains at 3 Pa, the uniformity of n_e is approximately 25% along chamber axis and n_e varies from 10^9 cm^{-3} to 10^7 cm^{-3} along the radial direction [7].

The following sterilization cycle was applied (refer to Figure 2):

1. The chamber was evacuated by rotary pump down to pressure lower than 1 Pa. Then, pure oxygen gas was injected into the chamber to increase the pressure to 100 Pa. Oxygen plasma was generated for 10min. Before the injection of calcium hydroxide solution vapor, the chamber was evacuated to 3 Pa for 20 min. Meanwhile, oxygen plasma maintained during this period.

2. Calcium hydroxide solution vapor was injected into the chamber after 30 min. of oxygen plasma ignition. The pressure of chamber increased to 100 Pa with duration of 5 min. Then reduced to 3 Pa and maintained for 5 min. This kind of pressure varying pulse was applied for two periods in the solution vapor plasma phase (the 2nd phase).

3. The same pressure sequence and plasma condition of the 1st phase was repeated after the 2nd phase.

Thus the period of plasma sterilization totally last 80 min. and the power density introduced into the chamber keep at the order of 10^{-3} W/cc. The ambient temperature of sterilization subjects was measured with thermo indicator, which was enveloped in Tyvek package with the inoculated substrates. Chemical indicator for hydrogen peroxide was also installed in the package.

Saturated calcium hydroxide aqueous solution was applied as vapor supply in the 2nd phase of sterilization cycle. Supply of vapor of calcium hydroxide aqueous solution into the chamber was performed through injection at one atmospheres pressure via a nozzle of needle valve. Heater mounted on the valve prevented freezing of the nozzle due to water evaporation. Shields covered on the sterilization subjects avoided the direct attachment of solution droplets to articles.

Bactericidal studies have been performed using spores of *B. atrophaeus* (ATCC#9372) and *G. stearothermophilus* (ATCC#7953) as the most conventional biological indicator for steam autoclave, EtO gas, dry heat and plasma sterilization. Suspension of spores, which density reaches 10^6 CFU/ml, was inoculated on substrates before subject to plasma. Doped silicon discs with surface of 4 cm^2 were used as spore deposition substrate, which were intended to void charging of the sample during plasma process and scanning electron microscope observation. However, they had been oxidized during the plasma sterilization procedure. Fortunately, the oxidation had not affected SEM observation. Stainless substrate was suggested to be a better

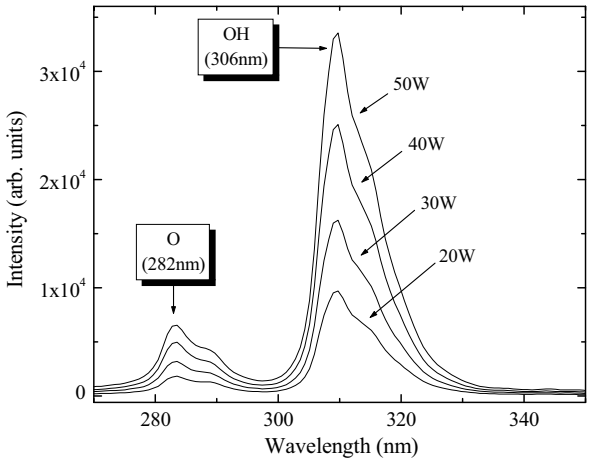

Fig.3 UV light emission spectra from plasma in the second phase of sterilization cycle. The introduced RF power ranged from 20 W to 50 W with 10 W intervals.

choice in the future. Normally, four discs are prepared for one set of experiment. For each data point four discs of samples were prepared. Three were used for microbiological analysis and the fourth for control or scanning electron microscopy.

The substrates are washed with a defined amount of sterilized water. A series dilution is made and 10ml of dilution was pipetted on medium sheets (Sanita-kun, Chisso Corp.). After 48 hour of incubation, the colony forming units (CFUs) are counted. Each CFU represents one survived microorganism. The plasma-induced reduction of CFUs is expressed logarithmically. The error margin of reduction value is accurate within one decade, taking into account the accuracy of the microbiological analysis.

3. Results and Discussion

The generation of oxygen and OH radicals was confirmed from light emission spectra using a photonic multi-channel analyzer and chemical indicator strip (ASP Johnson and Johnson).

Figures 3 shows typical UV light emission spectra from calcium hydroxide solution vapor plasma in the 2nd sterilization phase (refer to Figure 2). It was measured on the axis of the chamber using a photonic multi-channel analyzer, when the chamber pressure was 3 Pa. The peak of hydroxyl radical can be confirmed at 306 nm in the spectrum, while another peak around 282 nm is attributed to the emission from oxygen radical. Under this condition, the optical emission intensity of oxygen and OH radical increase gradually with increasing the RF power.

During initial stage of the 3rd phase of sterilization cycle (in about 10 min.), the intense peaks of hydrogen atom (H I) at 656 nm and 485 nm were observed under 100Pa, that indicate the dissociation of water molecules

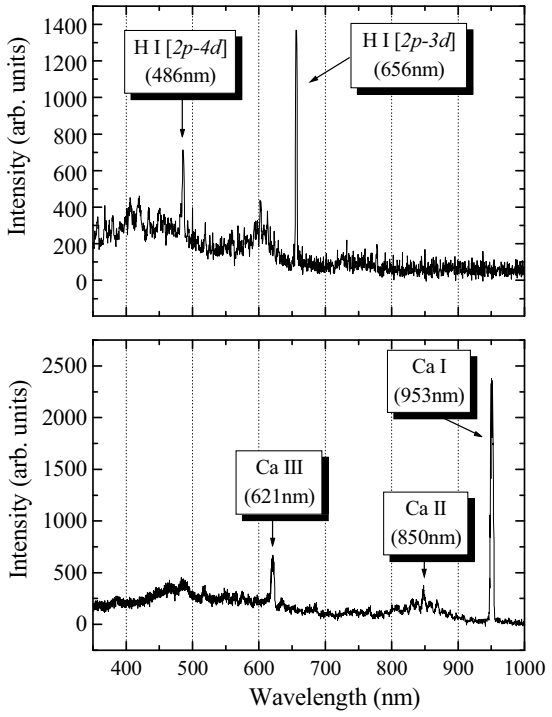

Fig.4 UV and visible light emission spectra from plasma in the third phase of sterilization cycle. Above spectrum was taken at the pressure of 100 Pa, while below was taken at 3 Pa.

in plasma (Figure 4. upper). Due to the gradual evacuation of residual solution, the emission peaks from calcium ion were observed under 3 Pa, after about 10min. launching of the 3rd phase of sterilization cycle. The emission was considered to be contributed to the residual calcium ion from calcium hydroxide solution, which had been deposited on plasma antenna and chamber during the 2nd phase.

In the 3rd phase of sterilization cycle, UV light emissions from oxygen and OH radical have also been observed in the spectra as shown in Figure 3., whereas the peak intensity descend gradually until the pressure of chamber decrease to 3Pa. These emissions due to residual water and calcium ion in the evacuated chamber availed the trace of residual calcium hydroxide solution. The emission results imply that the influence of water vapor should be considered at least in the initial 10 min. during the 3rd phase of sterilization cycle.

The emission spectra from the 3rd phase of sterilization cycle at 100 Pa (upper) and 3 Pa (lower) are illustrated in Figure 4 as typical spectra that indicate the water dissociation reaction (Formula 1 & Formula 2) and calcium ion activation in the plasma.

$$e + H_2O \rightarrow H + OH^- \quad (1)$$

$$O^* + H_2O \rightarrow OH + OH \quad (2)$$

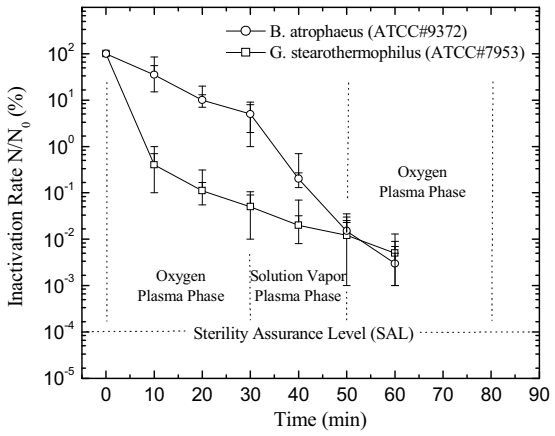

Fig.5 Survival curves for spores of *B. atrophaeus* and *G. stearothermophilus*, obtained by colony count method. The initial number of spores (N_0) is approx. 10^6. RF power introduced into the plasma was adjusted to 10^{-3} W/cc during 80 min. sterilization. The curve of *B. atrophaeus* reached sterility assurance level at 80 min.

Survival curve method had been used to evaluate inactivation efficiency during the sterilization cycle. Figure 5 illustrates the survival curves of the spores (number of survived spores *vs*. sterilization time). The decimal reduction time (D_1: D-value of the 1st phase plasma inactivation) approximate to 20 min. and 5 min. for *B. atrophaeus* and *G. stearothermophilus* respectively. During the 2nd phase plasma inactivation (calcium hydroxide solution vapor plasma), D_2 alter to 7 min. and 35 min. respectively. D_3 of both species remain similar to D_2 in the 2nd phase. *B. atrophaeus* had been successfully sterilized after the above mentioned 80 min. sterilization cycle, whereas *G. stearothermophilus* had not.

During the 1st phase of sterilization cycle, the survival curve of *G. stearothermophilus* has a two-slopes aspect. The D-value of the first 10 min. sterilization period was 30min. shorter compared to the consequent 20 min. sterilization period. It indicates that spores of *G. stearothermophilus* are more vulnerable to oxygen plasma, in the pressure range of 100Pa. This result is consistent with the previous experiment in oxygen RF plasma [7].

On the other hand, one can see that the decimal reduction time (D-value) of *B. atrophaeus* during the 2nd phase decreased significantly. This phenomenon can be interpreted due to hydrogen peroxide, which represents as an efficient sterilizing agent. It also follows from the figure that main role in sterilization of *B. atrophaeus* during the 2nd phase of sterilization cycle should be attributed to chemical etching performed by active particles like oxygen and hydroxyl radicals produced in plasma, associated with hydrogen peroxide.

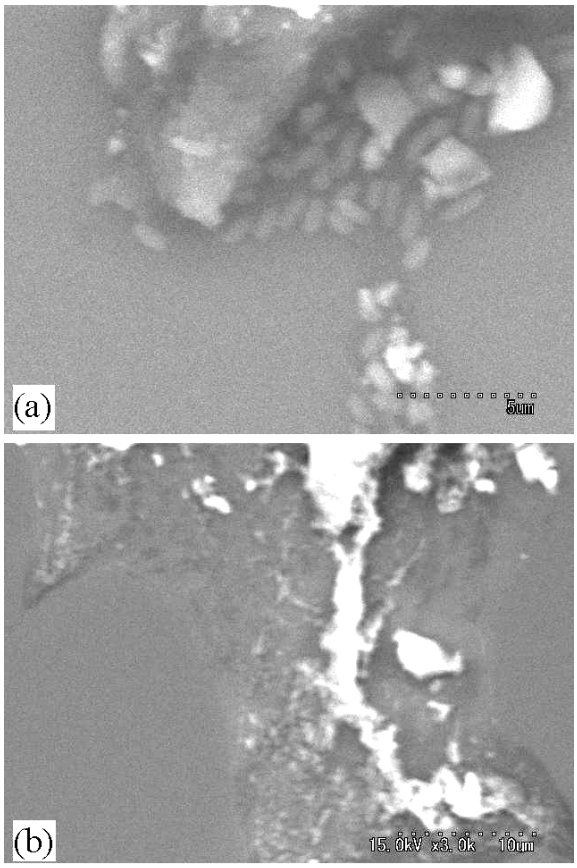

Fig.6 Scanning electron microscopy pictures of *B. atrophaeus* spores. (a) untreated, (b) treated with 80 min. sterilization cycle.

Figure 6 shows the morphological change of the *B. atrophaeus* spores. Fig.6 (a) illustrates the spores without any treatment deposited on substrate merged with debris. In Fig.6 (b), after calcium hydroxide solution vapor plasma treatment involved sterilization cycle, the spores are apparently dissolved due to the water-involved chemical etching in plasma.

4. Conclusions

The investigations of sterilization efficiency for *B. atrophaeus* (ATCC#9372) and *G. stearothermophilus* (ATCC#7953) wtih injection of calcium hydroxide solution vapor in plasma have been performed. The experiments have shown that *G. stearothermophilus* decreases sharply during the initial oxygen plasma sterilization phase (the 1st phase). While in the subsequent solution vapor plasma phase (the 2nd phase) it remains practically unimproved. The saturation of packaging paper by water vapor, which causes the decrease of its penetrability, may cause the D-value remained high even during the 3rd oxygen plasma phase in sterilization cycle.

The achieved experimental results show that:

1. The main role in sterilization of *B. atrophaeus*

spores by plasma in calcium hydroxide solution vapor is performed by hydrogen peroxide related reaction, rather than by oxidative radials produced in the plasma. Furthermore, it is confirmed by scanning electron microscopy that the significant improvement of inactivation rate to spores of *B. atrophaeus* is apparently due to water-involved chemical etching in the plasma.

2. The initial efficiency performed by pure oxygen plasma for sterilization of *G. stearothermophilus* spores is essentially higher than *B. atrophaeus* spores. Even though, *G. stearothermophilus* spores show higher resistance to chemical etching in the water-involved plasma.

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